GREEN SYNTHESIS AND CHARACTERIZATION OF BIMETALLIC AND TRIMETALLIC NANOPARTICLES USING PLANT EXTRACT AND EVALUATION OF ENHANCEMENT IN ANTIOXIDANT BIO-EFFICACY

A

SYNOPSIS

SUBMITTED FOR PARTIAL FULFILLMENT FOR THE AWARD OF

DOCTOR OF PHILOSPHY

IN CHEMISTRY

SUBMITTED BY

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(MAY, 2016)

ORIGIN OF PROBLEM

Among various metabolic reactions occurring in our body, oxidation reaction is highly important where the transfer of electron or hydrogen atoms from a molecule to an oxidizing agent occurs. Oxidation process, in addition to the formation of desirable bio-compounds also results into the formation of undesirable free radicals. These free radicals through chain reactions cause severe cell damage finally leading to several chronic diseases. Antioxidants are the compounds which terminate these free radical chain reactions or convert free radicals to less reactive species. Thus, they are also known as ***free radical scavengers.***

Realizing the medicinal value of antioxidant, a large number of synthetic antioxidants are prepared and available in the market, such as butylated hydroxyl anisole (BHA), ter-butyl hydro quinine (TBHQ), butylated hydroxyl toluene (BHT), propyl gallate (PG), and ethoxyquin (EQ). Recently, several side effects related to toxicity in liver, blood, stomach and lungs have been found to be associated with synthetic antioxidants. An urgent need of natural antioxidants which are safe and extracted from various parts of the plants has been realized.

Green Nanotechnology is a recent area of research interest and has significant application in industrial, environmental and pharmacological science. This approach can be successfully used in the green synthesis of nanoparticles using reduction potential of the herbal extract, simultaneously inducing phyto-chemical coating in them. Green nanoparticles embedded with phyto-constituents of specific medicinal importance behave as non-toxic, eco-friendly and are disease specific and, therefore, can be used towards design and development of target oriented substance with enhanced bio-efficacy.

Therefore, green nanotech approach in the area of pharmacological research is becoming a bright opportunity for the synthesis of environmentally friendly products and processes which are viable, desirable, scientific and technologically developed program in medicinal research.

INTRODUCTION

According to World Health Organization about 80% of the world population depends on traditional medicines for their primary health care needs, and it mostly includes the use of plant extracts and the bio-active components present in them (Khan *et al*., **2011**; Egwaikhide *et al*., **2007** and Krishnaiah *et al*., **2011**). Phytochemicals are the chemical compounds present in plants and their parts like fruits, seeds, bark and leaves etc and are capable of protecting us from various diseases. Phyto-chemicals can be classified into two categories primary and secondary metabolites. Primary metabolites include fatty acids, vegetative oils, carbohydrates, chlorophyll, proteins etc. Under environmental stress condition they are naturally changed into secondary metabolites which are of pharmacological importance such as terpenoids, alkaloids, flavonoids, polyphenols etc (Wadood *et al*., **2013**).

Table 1: List of secondary metabolites with their bio-efficacy (Hamid *et al*., **2010**)

Secondary Metabolites	Sources	Possible benefits
Flavonoids	Green tea, coffee apples ,citrus fruits	Antioxidant, cardiovascular diseases, arthritis, aging, cataract, memory loss, stroke, alzheimer's disease
Beta-carotene	Fruits, grains, oil and vegetables	Strong antioxidant , free radical quencher
Lycopene	Tomatoes	Antioxidant and anti-proliferative property
Vitamin E	Vegetable and grain oils, nuts, cereals,	Antioxidant property, anticancer, cardiovascular diseases, and neurological disorders
Vitamin C	Fruits, green vegetables,	Antioxidant, anti-atherogenic, anti-carcinogenic, and Immune-modulator
Omega-3 fatty acids	Vegetable oils, nuts, cereals, eggs, poultry	Anti-inflammation , anti- cancer, heart disease, stroke, memory loss, depression, and arthritis,
Alkaloids	*Poppy*, Solanaceae, Fabaceae plants	Antioxidant , antimicrobial, anti-inflammation, and anticancer
Polyphenol	Green tea, fruits, grains	Anti-cancer, anti-inflammation, and antioxidant
Terpenes	Cherries, citrus fruit peel, rosemary, etc.	Anticancer, antioxidant and antimicrobial

Antioxidative property is an important pharmacological bio-efficacy exhibited by secondary metabolites. Antioxidants are the compounds which act as inhibitors for the free radicals or ROS generate in the body or it is an electron deficient chemical compounds that have capacity to take electrons without causing any effect in its stability. These antioxidants retard or inhibit the cell damage. Oxidation reaction is a chemical reaction in which there is a transfer of electrons or hydrogen from one molecule to another molecule. These oxidation reactions produce free radicals. Free radicals have unpaired electron in an atomic orbital and due to this unpaired electron they are unstable and for stability they can either donate electrons or accept electron from other molecules like antioxidants. Antioxidants donate electrons to these free radicals and stabilize them and act as reducing agents without destabilizing themselves. Antioxidants are divided into two classes:

- Primary or chain breaking antioxidants
- Secondary or preventative antioxidants

Primary antioxidants either slow down or stop the initiation step with a radical or react with the peroxyl or alkoxyl radicals and inhibit the propagation step when present in small amounts while secondary antioxidants are compounds that slow down the rate of oxidation. This may be done either by removal of substrate or quenching of singlet oxygen.

Mode of Action of Antioxidants

Antioxidants on the basis of inactivation methods of radicals can be classified in two categories:

- Hydrogen atom transfer (HAT) reaction
- Electron transfer (ET) reaction

The mechanism and the efficiency of antioxidants depend on two factors which are bond dissociation energy and ionization potential. The hydrogen and electron transfer reaction occurs in molecules together so it sometimes gets very difficult to differentiate between them. Bond dissociation energy plays an important role in determining relative reactivity of HAT method. These reactions are fast and completed in seconds to minutes and are also solvent and pH dependent reactions.

$$AH + Y^{\cdot} \longrightarrow A^{\cdot} + YH$$

ET methods detect the potential of antioxidants to transfer electron to metals, carbonyls, and radicals and leads to the reduction of the compound.

$$M\ (III) + AH \longrightarrow AH^{\cdot} + M\ (II)$$

Deprotonation and ionization potential of the reactive functional group in ET methods determine the relative reactivity and that is why these reactions are pH dependent. As the pH increases ionization potential decreases in general. At lower pH, protonation of antioxidant compound and at high pH condition proton dissociation of phenolic compounds increases. These reactions comparatively to HAT reactions are slow (Karadag *et al*., **2009**).

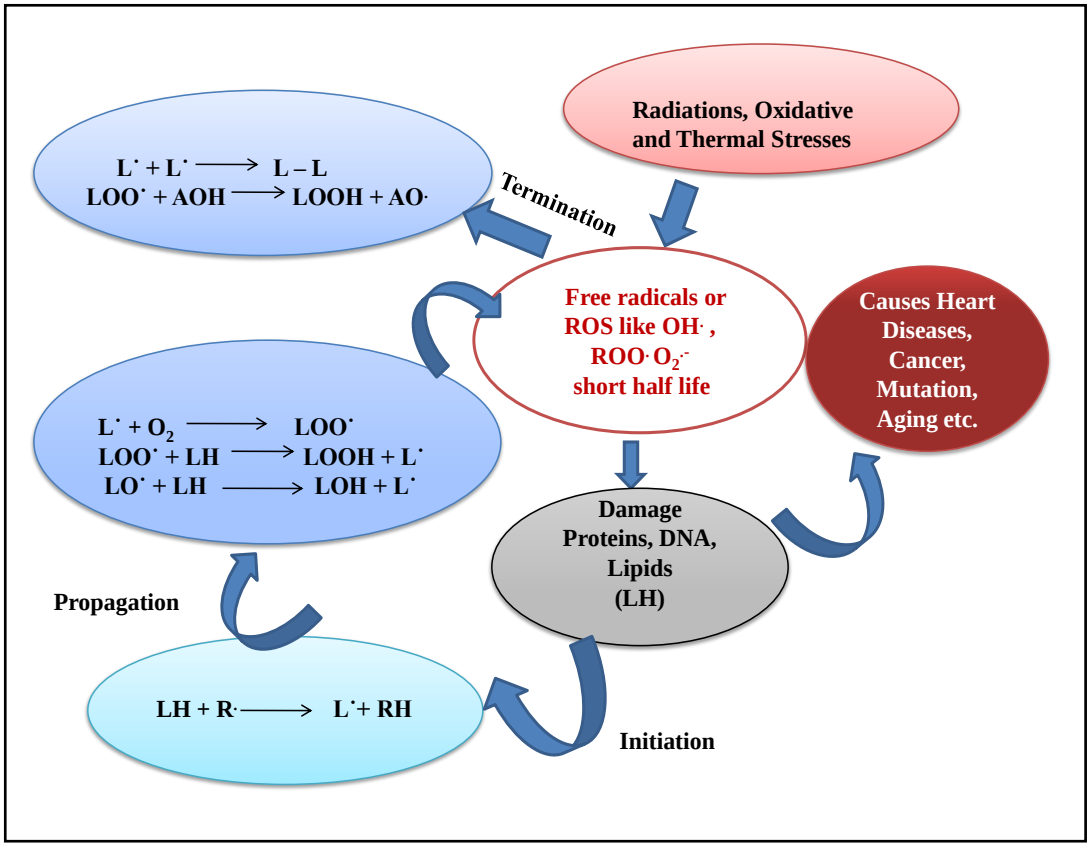

Radiations, Oxidative and Thermal Stresses
L˙ + L˙ ⟶ L – L
LOO˙ + AOH ⟶ LOOH + AO·
Termination
Free radicals or ROS like OH· , ROO· $O_2^{\cdot-}$ short half life
Causes Heart Diseases, Cancer, Mutation, Aging etc.
L˙ + O_2 ⟶ LOO˙
LOO˙ + LH ⟶ LOOH + L˙
LO˙ + LH ⟶ LOH + L˙
Damage Proteins, DNA, Lipids (LH)
Propagation
LH + R· ⟶ L˙ + RH
Initiation

Fig 1: Production of ROS and mechanism of damage of biomolecules (Antolovich *et al*., 2002 and Hamid *et al*., 2010).

Antioxidant can be categorized in two categories:

- ***Synthetic Antioxidants***
- ***Natural Antioxidants***

Synthetic antioxidants are those which are prepared in laboratories like butylated hydroxyl anisole (BHA), ter-butyl hydro quinine (TBHQ), butylated hydroxyl toluene (BHT), and propyl gallate (PG), ethoxyquin (EQ) are used as preservatives in food (Yao *et al*., **2013)**. But all these synthetic antioxidants are found to be toxic and carcinogenic in nature. So, these antioxidants are restricted to use in food and food products as preservatives. While natural antioxidants are those which present in plants and their parts

are such as fruits, vegetables, and grains like terpenoids, alkaloids, flavonoids, polyphenols etc (Kaur *et al.*, **2001**; and Shahidi **2000**).

There are many natural antioxidants which are also present in our body such as glutathione, alpha-lipoic acid, coenzyme Q, ferritin, uric acid, bilirubin, metallothionein, L-carnitine, melatonin, enzymatic superoxide dismutase (SOD), catalase (CAT), glutathione peroxidases (GPXs), thioredoxins (TRX) and peroxiredoxins (PRXs) which makes free radicals less reactive. But if these reactive oxygen species present in high concentration then it leads to oxidative stress. Oxidative stress is a condition in which antioxidant which are present in the body are not sufficient to completely inactivate the free radicals which leads to cancer, diabetes, atherosclerosis, inflammatory diseases, and ageing. It can also damage cells, DNA, lipid and protein peroxidation.

The significance of bioactive phytochemicals in counteracting free radicals is gaining credence in the present prospective. Phyto-antioxidants from medicinal plants are of particular importance because they are proved to be highly beneficial for several diseases. Researchers are now more focused on extraction of antioxidants from plant and its parts like leaves, root, fruits, flower bud, and bark etc (Shahid *et al.*, **2013**; Dasari *et al*., **2011** and Romano *et al*., **2009**).

Table 3: List of plant exhibiting antioxidant activity

Plant Name	Bioactive Principle	Bioefficacy	References
Acacia confuse	Polyphenols	Antioxidant	Chang *et al*., **2001**
Licania licaniaeflora	Flavonoids	Antioxidant	Braca *et al*., **2002**
***Pimpinella anisum* L.**	Polyphenols	Antimicrobial	Gulcin *et al*., **2003**
Zingiber officinale	Gingerol	Antioxidant	Masuda *et al*., **2004**
Syzygium cumini	Vitamins, phenolics or tannins and	Antioxidant	Banerjee *et al*., **2005**
***Ocimum basilicum* L.**	Eugenol, thymol, carvacrol	Antioxidant	Lee *et al*., **2005**
***Psidium guajava* L.**	Phenols and ascorbic acid	Antioxidant	Yan *et al.*, **2006**
***Lamium purpureum* L.**	Polyphenols	Antioxidant	Matkowski and Piotrowska **2006**
***Morus indica* L.**	Polyphenols	Antioxidant	Arabshahi-Delouee, Urooj **2007**
Rosmarinus Officinalis	Tocopherols, flavonoids	Antioxidant	Erkan *et al*., **2008**
Ocimum minimum	Polyphenols	Antioxidant	Hakkim *et al*., **2008**
Ballota nigra	Flavonoids, and diterpenes	Antioxidant	Matkowski *et al*., **2008**
***Brassica oleracea* L.**	Phenolics or vitamin C	Antioxidant	Borowski *et al*., **2008**
Cuminum cyminum	Essential oils	Antioxidant	Hassan El-Ghorab *et al*., **2010**
Polyalthia longifolia	Terpenoids	Antioxidant	Dasari *et al*., **2011**

Plant Name	Bioactive Principle	Inefficacy	References
Allium sativum	Essential oil and allicin	Antioxidant	Lawrence **2011**
Lantana camara	Polyphenols	Antioxidant	Mahdi-Pour *et al*., **2012**
B. xbuttiana	Polyphenols	Antioxidant	Figueroa *et al*., **2014**
***Mangifera indica* L.**	Polyphenols	Antioxidant	Martínez *et al*., **2012**
Elettaria cardamomom	Essential oil, flavones and oleoresins	Antimicrobial Antioxidant	GnanaJobitha *et al.*, **2012** Singh *et al.*, **2008**
Syzygium aromaticum	Eugenol and eugenyl Acetate	Antioxidant, Antibacterial	Wojdyło *et al*., **2007** Lee, Shibamoto **2001**
Phyllanthus emblica	Alkaloids, flavonoids, proteins, saponins and Tannins.	Antioxidant, Antitumor	Sumalatha **2013**
Camellia sinensis	Polyphenols and caffeine	Antioxidant, Anticancer	Thasleema **2013**
Trachyspermum ammi	Tannins, glycosides, protein	Antimicrobial	Sahu *et al*., **2015**
***Piper nigrum* L.**	Thiobarbituric acid	Antioxidant	Suhaj *et al*., **2006** Mallick *et al.*, **2016**
T. foenum-graecum	Flavonoids and Phenolic compound	Antioxidant	Mallick *et al.*, **2016**
Hammada scoparia	Alkaloids	Antibacterial, Antioxidant	Bouaziz *et al.*, **2016**

Nanotechnology is an important interdisciplinary area of research and has been involved in the development of excellent procedures and products in chemicals, electronics, agriculture, space and pharmaceutical industries. However, several issues like health, cost effectiveness, environmental and safety risks are found to be associated with recent Nanotechnological applications particularly, in pharmaceutical and biological systems. A debate has also been generated about the **Greenness of Nanotechnology.**

Green nanotechnology is the new dimension and intersection of Nanotechnology with Green Chemistry. Green chemistry is an approach to chemical synthesis, considering production and management of wastes, toxicity, energy use and most importantly cost effectiveness. Synthesis of nanoparticles using plant extracts is the eco-friendly, economical and non- toxic production of biogenic metal nanoparticles. Chemical partnership of various phytochemicals like flavonoid, terpenoids, poly phenolic etc. containing functional groups such as (COOH, C=O, NH_2, SH and OH) provide powerful **Synergistic Chemical Reduction** potential. Phytochemicals or Secondary metabolites of pharmacological importance not only reduce metal but also provide a coating of phytochemicals on the freshly prepared nanoparticles and thus adding plant medicinal properties. Such plant mediated Nano particles are known as **Precious Metal Nanoparticles Embedded with Phyto extracts.** Further, coating of phytochemicals on the nanoparticles not only induces medicinal properties but delivers robust shielding and prevents aggregations of particles, keeping them in Nano state (stability). Thus, Green Nanotechnology provides an unprecedented process

for the production and stabilization of precious nanoparticles in a singular green process, opening the gate of wide applications in Nano medicine. The reduction of Au, Ag, and Pt is expected to occur through the oxidation of antioxidant's hydroxyl to carbonyl groups according to the following reactions (Anuradha *et al*., **2015** and Lin *et al*., **2006**).

$[AuCl_4]^- + 3R\text{-}OH \longrightarrow Au^0 + 3R{=}O + 3H^+ + 4Cl^-$
$AgNO_3 + R\text{-}OH \longrightarrow Ag^0 + R{=}O + H^+ + NO_3^-$
$[PtCl_6]^{-2} + ROH \longrightarrow [PtCl_4]^{-2} + RCHO + 2H^+ + 2Cl^-$
$[PtCl_4]^{-2} + 2ROH \longrightarrow Pt^0 + RCHO + 4H^+ + 4Cl^-$

Multimetallic catalytic systems can potentially achieve chemical transformations that are not possible with monometallic catalysts because different components of the catalyst have a particular function in the overall reaction mechanism (Li *et al*., **2013**; Gopinath *et al*., **2013**; Rao *et al.*, **2015** and Singh *et al.*, **2013**).

Table 4: List of Bimetallic/Trimetallic Nanoparticles from Plant Extract

Plant Name	Bi/Trimet-allic Nanoparticles	Reducing Agent	References
Cacumen platycladi	Au–Pd	Flavonoids	Zhan *et al.*,**2011**
***Potamogeton pectinatus* L.**	Au–Ag	Flavones and proteins	Hamid *et al*., **2013**
Cacumen platycladi	Au–Ag	Polyphenols, reducing sugars,	Zhang *et al.*, **2013**
Azadirachta indica	Au –Ag shell	Reducing sugars, terpenoids	Shankar *et al.*, **2004**
Swietenia mahogany	Au/Ag alloy	Polyhydroxy limonoids	Mondal *et al.*, **2011**
Diopyros kaki	Au/Ag	Not reported	Song and Kim **2008**
Punica granatum	Au–Ag	Phenolic compounds	Kumari *et al.*, **2015**
Piper pedicellatum	Au–Ag	Catechin and gallic acid	Tamuly *et al.*, **2013**
Camellia sinensis	Fe/Pd	Polyphenols	Smuleac *et al.*, **2011**
Volvariella volvacea	Au–Ag	Polysaccharide	Philip **2009**
Terminalia arjuna	Ag/Au	Arjunetin, leucoanthocyanidins	Gopinath *et al.*, **2013**
Anacardium occidentale	Au−Ag	Polyols and proteins	Sheny *et al.*, **2011**

Table 5: List of Microwave Assisted Synthesis of Bimetallic/Trimetallic Nanoparticles

Bimetallic/Trimetallic Nanoparticles	Stablizing Agent	Reducing Agent	References
Cu–Ag	Starch	Ascorbic acid	Valodkar *et al*., **2011**
Au–Ag	Glucose	glucose	Raveendran *et al*., **2006**
Au/Pt/Ag	trisodium citrate	Not used	Karthikeyan *et al.*, **2012**
Au-Pt-Ag	trisodium citrate	PVP	Karthikeyan *et al*., **2013**

OBJECTIVES

The present work will be carried out with the focus on the synthesis and characterization of monometallic, bimetallic and trimetallic (*Au, Ag, Pt*; Au-Ag, Au-Pt, Ag-Pt) and (Au-Ag-Pt) nanoparticles using spice extract (clove) for the evaluation of enhancement in the antioxidant activity measured by DPPH and Fenton assays.

Objectives with Rationale

S.NO.	OBJECTIVES	JUSTIFICATION
1.	Synthesis of bimetallic and trimetallic nanoparticles	*Design and synthesis of bimetallic and trimetallic nanomaterials have recently attracted considerable attention of scientific community as they are supposed to have better catalytic activity, selectivity, and stability over monometallic nanomaterials. (Li et al., **2013**; Gopinath et al., **2013**; Rao et al., **2015** and Singh et al., **2013**).*
2.	Selection of Au, Ag and Pt metals	*Since ancient time gold and silver metals are used in medicines in the form **Bhasms** as they themselves have medicinal properties. Platinum shows very good catalytic property and exhibits free radical scavenging activity and suppress the propagation of lipid peroxidation (Antioxidant activity; Lee et al., **2015**).*
3.	Selection of Clove Extract as natural reducing agent	*Clove extract contains maximum total phenolic content (**16,550 mg GAE/g) and** oxygen radical absorbance capacity (**290,283 mmol TE /100 g)** (Charles **2013**).*
4.	Use of citric acid containing fruit as supporting reducing agent for the preparation of trimetallic nanoparticles	*Synthesis of trimetallic nanoparticles require strong reducing agent and prepared synthetically. Attempt will be made to use combination of two plant extract for this purpose as green synthesis. (Rao and Paria **2015**).*
5.	Expected Enhancement in Antioxidant Bio-efficacy in the case of precious metal nanoparticles embedded with phytochemicals compared to native extract	*The generation of remarkable optical properties owing to localized surface plasmon resonance, high surface area to volume ratio of nanoparticles and dense loading of functionalities (phytochemical coating) provides enhanced bio-efficacy and stability(Ahmed et al., **2016** and Baker et al., **2013**).*

PLANT UNDER STUDY

Clove

Classification

Kingdom : Plantae
Order : Myrtales
Family : Myrtaceae
Genus : *Syzygium*
Species : *aromaticum*

Chemical constituents (Lee *et al*., **2001**; Abdel-Wahhab *et al.*,**2005**)

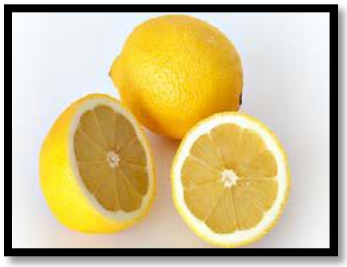
Eugenol
Beta-caryophyllene,
Crategolic acid, Tannins
Gallotannic acid
Methyl salicylate
Flavonoids

Citric Acid contained in some fruits

Amla

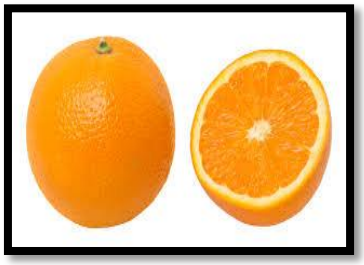
Classification

Kingdom : Plantae
Order : Geraniales
Family : Euphorbiaceae
Genus : *Emblica*
Species : *officinalis*



Lemon

Kingdom : Plantae
Order : Sapindales
Family : Rutaceae
Genus : *Citrus*
Species : *limon*



Orange

Kingdom : Plantae
Order : Rosidae
Family : Rutaceae
Genus : *Citrus*
Species : *sinensis*

METHODOLOGY

*Extraction of the Plant Material (*clove/ cinnamon/ cumin)

Selected plant material will be washed with water, dried and cut into small pieces. Powder of clove extract will be soxhaleted using aqueous alcoholic solvent. Extract will be concentrated in the known volume using rota vapor and dried by purging nitrogen and weighed. Different concentration will be prepared from the stock solution of spice extract.

Phytochemical analysis

Aqueous alcoholic extract of spice (clove) will be analyzed using LC-MS and GC-MS for characterization of various phytochemicals present in it.

Estimation of Total Phenolic and Flavonoids

Total flavonoids and phenolic will be quantitatively measured using standard methods (Moukette *et al*., **2014**).

Synthesis of Monometallic nanoparticles (Au, Ag and Pt)

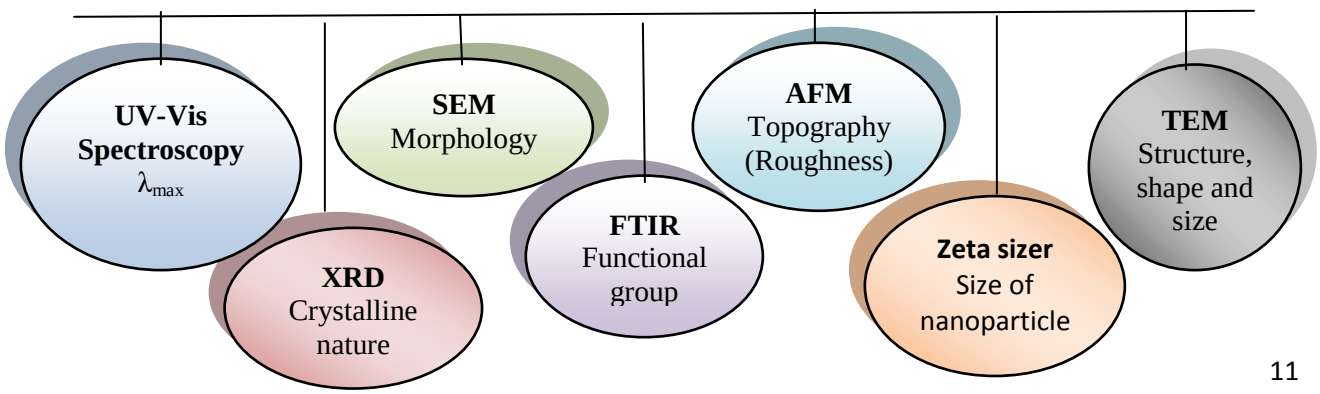
Appropriate volumes (to be optimized) of clove extract (5– 50 ml) at different pH (4, 7.5 and 9) will be added to appropriate amount (to be optimized) to $HAuCl_4$ (Chloroauric Acid; 0.001M). Reaction mixture will be sonicated and observed for color change. Color change gives indication of the formation of nanoparticle. Silver and platinum monometallic nanoparticles using appropriate volume of Silver Nitrate ($AgNO_3$; 0.001M) and Chloroplatinic acid (H_2PtCl_6; 0.001M).

Synthesis of Bimetallic and Trimetallic Nanoparticles (Au-Ag, Au-Pt, Ag-Pt and Au-Ag-Pt)

Monometallic nanoparticle's solution of gold, silver and platinum will be mixed in different ratios (to be optimized) for the synthesis of bimetallic nanoparticles. Reaction mixture will be sonicated and observed for color change. For the synthesis of trimetallic nanoparticles, an additional plant extract (amla/lemon/orange) will also be used to enhance reduction capacity.

Characterization of nanoparticles

Monometallic, bimetallic and trimetallic nanoparticles formed will be characterized using following techniques:



Determination of antioxidant activity

DPPH (2'2-Diphenyl-1-picrylhydrazyl) assay (Kaur *et al*., **2006**)**:**

Free radical scavenging activity of extract can be determined by DPPH assay. The reaction mixture of dilution series (10-100 mg/ml) taking different volumes of extract (10-100µl) will be incubated with DPPH (3ml ; 0.15 mM) solutions in methanol. The solution will be allowed to stand for 30 minutes at room temperature. Extracts when reacted with DPPH, a stable purple colored free radical will convert into colorless compound α-α diphenyl β-picryl hydrazine will be formed. The extent of decoloration indicates the amount of DPPH scavenged. The absorbance will be measured at 517nm. Ascorbic acid will be used as a reference antioxidant. The percent inhibition of DPPH will be calculated using the formula:

[(C-T)/C]X100

C is the absorbance of control and **T** is the test sample.

Fenton assay **(**Kunchandy *et al*., **1990)**:

The reaction mixture containing different dilution series (10-100 mg/ml) taking different volumes of extract (10-100µl) will be incubated with deoxyribose; (3.75 mM), H_2O_2; (1mM), $FeCl_3$; (100µM), EDTA; (0.1 mM) and ascorbic acid; (100 µM) in phosphate buffer having pH (7.4). The reaction will be terminated by thiobarbituric acid (1ml; 1% w/v) and trichloro acetic acid (1ml; 2% w/v) and heated in a boiling bath for about 15 minutes. The pink chromogen which will be formed eventually results in the formation of thiobarbituric acid reactive substance (TBARS). The absorbance will be measured at 532 nm. The percent inhibition of OH generated will be calculated using the formula:

[(C-T)/C]X100

C is the absorbance of control and **T** is the test sample.

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